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Method for lowering the effects of sequence variations in a diagnostic hybridization assay, probe for use in the assay and assay

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METHOD FOR LOWERING THE EFFECTS OF SEQUENCE VARIATIONS IN A DIAGNOSTIC HYBRIDIZATION ASSAY, PROBE FOR USE IN THE ASSAY AND ASSAY

The present invention relates to a method for lowering the effect of sequences variations in diagnostic hybridization assays that use a nucleic acid probe to detect an amplified nucleic acid analyte. The invention further relates to the diagnostic assays thus obtained and to probes for use in such assays.

Many diagnostic assays are based on the amplification of a nucleic acid molecule or part thereof with the help of primers and the detection of the amplified material by means of a probe. Under the appropriate reaction conditions, the primers hybridise to the analyte to be detected and initiate amplification of the target sequence. This will lead to the generation of amplicons.

Various amplification techniques are meanwhile developed, such as PCR, LCR, NASBA, TMA and SDA, and well known to the person skilled in the art. During or after amplification of the analyte or part thereof, the presence or amount of amplicons generated should be detected. This can be done with various known techniques such as separation of the sample on a gel with subsequent blotting and probing. This can only be done after the amplification is finished.

In a homogeneous procedure, amplification and detection occur without separating the reaction components. Amplicons are detected in the course of the amplification. Thus, the generation of amplicons can be monitored real-time and the data thus obtained can be used to determine the presence or absence or the amount of the amplicon. One type of

probe that is very useful in such homogeneous techniques is the molecular beacon.

Molecular beacons are single-stranded oligonucleotides having a stem-loop structure. The loop portion contains the sequence complementary to the target nucleic acid (either DNA or RNA). The stem is formed due to hydridisation of the complementary sequence of the 3' end with the 5' end. The stem can be unrelated to the target and is double-stranded. One arm of the stem is labelled with a fluorescent dye (fluorophore), whereas the other one is coupled to a quenching molecule. In the stem-loop state the probe does not produce fluorescence because the energy of the fluorophore is transferred to the quenching molecule. When the molecular beacon hybridizes to the target the stem-loop structure is lost and the quencher and fluorophore are separated. At that stage the fluorescence emitted by the fluorophore can be detected and quantified.

The amplicons generated in the amplification reaction can be detected quantitatively or qualitatively. In the former case the amount of amplicons generated is quantified. In a qualitative assay only the presence or absence of the analyte is determined.

Sequence variations (polymorphisms) in the analyte can lead to an under-quantification thereof. Also in case of qualitative assays sequence variations can result in false negatives. It is generally assumed that this is caused by mismatches between the analyte and the primers used to amplify the analyte or by the structure of the analyte, which causes primers not to bind.

It is known that various polymorphic pathogenic strains of viruses exist as for example for the analytes HIV,

CMV, HSV etc. These polymorphic strains differ from each other usually by one or more nucleotides. When a primer differs from the analyte it does not fit very well which leads to a reduced amplification. When the analyte is not linear but has a particular structure, it is less accessible by the primer, which in turn also leads to a reduced amplification.

In addition, sequence differences between the amplified target sequence of the analyte and the probe used for detection further lower the efficiency of detection. Analytes with polymorphisms, are thus less well detected than analytes that match perfectly with the consensus sequence of the probe. However, detection is usually performed at lower temperatures than the amplification, and therefore the negative effect of mismatches between probe and target is expected to be far less than is caused by differences between primer and analyte.

The probe can be optimised to fit known polymorphisms. However, in the case of unknown polymorphisms this is not possible. This is particularly a problem since new, unknown polymorphisms are continuously generated, which hamper a reliable detection or quantification especially in the case of HIV.

In the research that led to the invention it was now found that in a NASBA amplification reaction of a (clinical) sample of an HIV virus with an unknown polymorphism no signal or lower signals were generated in the Molecular Beacon detection system. However, surprisingly amplicons could be detected when the Molecular Beacon probe was modified such that the melting temperature of the probe with the analyte was increased. This led to the unexpected conclusion that

amplicons were indeed produced but not or only partially detected.

It is therefore the object of the present invention to lower the effects of sequence polymorphisms in an hybridization assay by manipulation of the affinity between the probe and the analyte. This is achieved according to the invention by introduction into the probe of one or more nucleotides and/or nucleotide analogues that result in an increase of the affinity between the analyte and the probe.

The invention thus relates to the use in a diagnostic hybridization assay of a probe for lowering the effect of sequence variations in a nucleic acid analyte, which assay comprises the steps of contacting a set of primers and a sample containing the nucleic acid analyte to amplify the analyte and detecting the amplified analyte or its complement by means of the probe, wherein the probe comprises one or more nucleotides and/or nucleotide analogues that have an affinity increasing modification.

The binding between a probe and a target is an equilibrium between unbound target and unbound probe on the one hand and the duplex between the two on the other. This equilibrium is described by the melting temperature (Tm) of the duplex which is defined herein as the temperature at which 50% of the probe is bound to the target nucleic acid in a duplex. Shifting the equilibrium towards the duplex can be achieved by an increase in the melting temperature. In an assay this will lead to equal quantification of perfectly matching analytes, a better (i.e. higher) quantification of analytes containing polymorphisms in the sequence that is complementary to the probe and an improved detection of very small amounts of (polymorphic) analytes.

As used herein the term "probe" is intended to comprise a stretch of nucleotides hybridising to the target. Preferably the hybridising part is a stretch of 10-50, more preferably 15-35, most preferably 15-30 nucleotides.

The term "affinity increasing" means that the melting temperature of a duplex between a probe comprising an affinity increasing modification and an analyte is increased as compared to the melting temperature between the analyte and the unmodified probe.

The nucleotides or nucleotide analogues having a modification that increases the affinity of a probe containing them to DNA or RNA targets are preferably selected from the group consisting of 2'-O-derivatized nucleotides, locked nucleic acids (LNAs) and peptide nucleic acids (PNAs). In the case of 2'-O-derivatized nucleotides it is preferably a 2'-O-methyl-nucleotide.

The probe according to the invention is preferably a so-called molecular beacon (MB). These probes recognize their targets with higher specificity than linear probes and can easily discriminate targets that differ from one another by a single nucleotide. By the introduction of one or more nucleotides and/or nucleotide analogues that have an affinity increasing modification, and more particularly in the loop of the molecular beacon, the sensitivity to polymorphisms is lowered because the affinity of the probe to the polymorphic analyte is increased and more accurate results are obtained in the assay. Thus, the modified molecular beacon becomes a very versatile tool for lowering the dependency towards sequence variation of the target in a homogenous assay.

The modified nucleotides can be used to synthesize the entire probe or to make a chimeric probe in which only a number of nucleotides is replaced by a modified nucleotide.

The amount of modifications that is minimally necessary to neutralize the mismatches is dependent on the amount of differences between the consensus sequence of an analyte and the sequence of the analyte to be detected. In general, it can be stated that the amount of modifications should be such that the melting temperature of the duplex between the probe and the analyte to be detected should lie above the detection temperature, i.e. the temperature at which the detection is performed.

The amount of modified nucleotides in a probe also depends on the type of modification used. The increase in Tm (the melting temperature of the probe with its target) upon introduction of a single LNA nucleotide in the probe is much higher as compared to the effect of a single 2'-O-methyl-nucleotide. In the Examples it is e.g. demonstrated that the introduction of two LNA nucleotides in the probe increased the Tm with 15°C, while twelve 2'-O-methyl-nucleotides were needed to obtain the same increase in Tm.

Analytes to be detected, such as HIV, may contain so-called "hot spots" that are prone to mutations and thus leading to polymorphisms. When the position of those hot spots or other positions of mismatches of the analyte and the probe are known, such as in the case of known isolates, it is preferred to arrange the nucleotides or nucleotide analogues having an affinity increasing modification around the position of the polymorphism, preferably on conserved positions.

The present invention further relates to a hybridization assay using a nucleic acid probe to detect a

nucleic acid analyte, wherein the probe comprises one or more nucleotides and/or nucleotide analogues that have an affinity increasing modification. The modified nucleotides and/or analogues thereof used in the probe are as defined above.

The hybridization assays of the invention may be of any kind in which a nucleic acid probe is used to detect a nucleic acid analyte. Such assays may be based on the detection of amplified analytes, such as in PCR-, TMA- or NASBA-based assays. However, the probe can also be used in arrays. The invention can be used both in quantitative and qualitative diagnostic assays in which sequence polymorphisms of the target influence the reliability of the assay.

Diagnostic assays that benefit from the invention are for example assays for detecting viruses, bacteria and other biomarkers as for example HIV, HBV, HCV, HSV, CMV, Ebola, legionella, mycoplasma, TNF-α, ER-α etc., as long as these diagnostic assays are characterised in such a way that they make use of the hybridisation between the analyte of interest and the modified oligonucleotide.

The invention also relates to a probe that comprises one or more nucleotides and/or nucleotide analogues that have an affinity increasing modification. The probe is preferably a molecular beacon.

In this application the terms "analyte", "amplicon" and "target" or "target sequence" may be used interchangeably. The analyte is the original nucleic acid molecule to be detected. The target sequence is the part of the analyte that is amplified by means of the primers. The amplification leads to formation of amplicons, which are the nucleic acid molecules that are physically detected by hybridisation to the

probe. The sequence of the amplicons is the same or complementary to the target sequence within the analyte.

The present invention will be further illustrated in the Examples that follow and which are not intended to limit the invention in any way.

EXAMPLE 1

Effect of the replacing nucleotides in a probe with 2'-O-methyl derivatives on the Tm of the duplex

In the quantification experiments described below, standard NASBA amplification conditions have been used to obtain amplified material. In every experiment all reaction conditions were identical and the only variation was in the type of probe (Molecular Beacon (MB)) that was used. As a model system, a set of well characterized viral lysates that represented most of the different subtypes of HIV-1 was used.

Melting temperatures (Tm's) of the amplified material with the MB's were determined by measuring the fluorescence intensity of the reaction mixtures as a function of the increasing temperature. From these melting curves the Tm of the RNA-MB complex (in the presence of excess RNA) could be determined.

Quantification results were obtained by using specially developed software, that allows curve fitting of the real time amplification curves. The Tm's of the different MB's used are shown in Table 1.

Table 1

Name	Tm loop DNA	Tm loop RNA	position
Ref MB	58.6	48.6	A T C A A T G A G G A I G C T G C A G A I T G G G A
Me-1	64.7	53.8	A T C A A T G A G G A I G C T G C A G A I T G G G A
Me-2	58.7	62.1	A T C A A T G A G G A I G C T G C A G A I T G G G A
Me-3	59.2	>65	A T C A A T G A G G A I G C T G C A G A I T G G G A
Me-7	59.4	>65	A T C A A T G A G G A I G C T G C A G A I T G G G A

2'-O-Me nucleotide

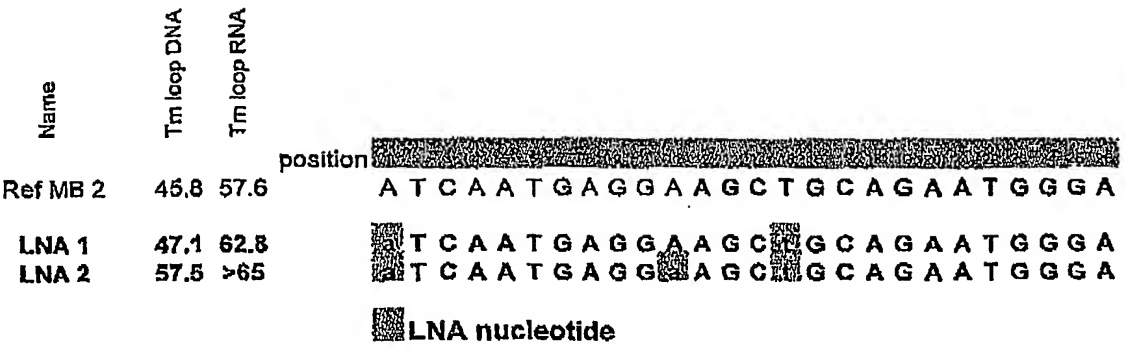
As can be seen from the data in Table 1, the introduction of 2'-O-methyl derivatives leads to an increased affinity (higher Tm loop) between target RNA and the MB loop. As expected, this increase is less pronounced for the DNA-MB complex, since 2'-O-methyl derivatives are known to bind stronger with RNA than DNA targets.

EXAMPLE 2

Effect of replacing nucleotides in a probe with LNAs on the Tm of the duplex

In Table 2, the effect of two and three LNA building blocks on the melting temperature has been investigated. From this Table it can be seen that LNA nucleotides increase the Tm of both DNA and RNA complexes. Also it can be seen that the effect on the Tm per modification is much higher as compared to the 2'-O-methyl nucleotides.

Table 2

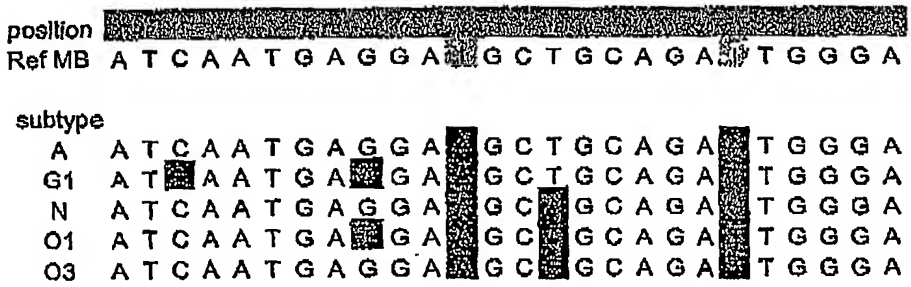


EXAMPLE 3

Detection of different HIV-1 isolates with the modified MBs

The HIV-1 isolates which have been selected to act as a model system to investigate the effect of sequence polymorphisms in the target RNA sequence are shown in Table 3. These materials were available as viral lysates and have been used in a NASBA amplification with the MB's of example 1 and 2.

Table 3



The quantification results that were obtained with four of the MB's as compared to our reference MB are shown in Table 4.

Table 4

subtype	ref MB	LNA2	Me-2	Me-3	Me-7
A	6.5	6.5	6.5	6.5	6.5
G	4.7	5.9	6.4	6.6	6.6
N	4.3	4.6	5.2	5.0	5.3
O1	3.3	4.9	5.4	5.8	5.9
O3	4.9	5.8	6.3	6.2	6.2

Since the A-subtype fits perfectly with all four MB's, all data are normalised based on this subtype. The other subtypes have been selected since they show sequence variation with the loop binding region of the MB (see Table 3). As can be seen, the highest quantifications for all five subtypes have been observed with the Me-7 MB derivative.

To investigate the relationship between the better quantification and the Tm loop value (°C) of the different MB's, the latter were determined for the duplex between the molecular beacon derivatives and the NASBA amplicons. This yielded the results shown in Table 5.

Table 5

subtype	ref MB	LNA2	Me-2	Me-3	Me-7
A	48	65	62	64	65
G	45	52	58	63	65
N	42	55	53	55	60
O1	27	44	44	43	53
O3	40	52	54	56	60

As can be seen from Tables 4 and 5 a clear correlation can be observed between a higher Tm loop (between the amplicon and the MB) and a higher quantification.

CLAIMS

1. Use in a diagnostic hybridization assay of a probe for lowering the effect of sequence variations in a nucleic acid analyte, which assay comprises the steps of contacting a set of primers and a sample containing the nucleic acid analyte to amplify the analyte and detecting the amplified analyte or its complement by means of the probe, **characterized in that** the probe comprises one or more nucleotides and/or nucleotide analogues that have an affinity increasing modification.

2. Use as claimed in claim 1 wherein the diagnostic assay is for assessing the presence of the analyte in the sample.

3. Use as claimed in claim 1 wherein the diagnostic assay is for assessing the amount of analyte present in the sample.

4. Use as claimed in any one of the claims 1-3 wherein the diagnostic assay is a homogeneous assay.

5. Use as claimed in any one of the claim 1-3 wherein the diagnostic assay is a heterogeneous assay.

6. Use as claimed in any one of the claims 1-5, wherein the nucleotides or nucleotide analogues having an affinity increasing modification are selected from the group consisting of 2'-O-derivatized nucleotides, locked nucleic acids and peptide nucleic acids.

7. Use as claimed in claim 6, wherein the 2'-O-derivatized nucleotide is a 2'-O-methyl-nucleotide.

8. Use as claimed in claims 1-7, wherein the probe is a molecular beacon.

9. Use as claimed in claim 8, wherein the molecular

beacon comprises the modified oligonucleotides or analogues thereof in the loop.

10. Probe for use in a diagnostic hybridization assay, comprising the steps of contacting a set of primers and a sample containing a nucleic acid analyte to amplify the analyte and detecting the amplified analyte by means of the probe, which probe comprises one or more nucleotides and/or nucleotide analogues that have an affinity increasing modification.

11. Probe as claimed in claim 10, wherein the nucleotides or nucleotide analogues having an affinity increasing modification are selected from the group consisting of 2'-O-derivatized nucleotides, locked nucleic acids, peptide nucleic acids.

12. Probe as claimed in claim 11, wherein the 2'-O-derivatized nucleotide is a 2'-O-methyl-nucleotide.

13. Probe as claimed in any one of the claims 10-12, wherein the probe is a molecular beacon.

14. Probe as claimed in claim 13, wherein the molecular beacon comprises the modified oligonucleotides or analogues thereof in the loop.

15. Kit for performing a diagnostic amplification assay, comprising the appropriate primers, polymerase(s) and reagents for performing amplification of an analyte to be diagnosed and a probe as claimed in claims 10-14 for detecting the amplified analyte.

ABSTRACT

The present invention relates to the use in a diagnostic hybridization assay of a probe for lowering the effect of sequence variations in a nucleic acid analyte, which assay comprises the steps of contacting a set of primers and a sample containing the nucleic acid analyte to amplify the analyte and detecting the amplified analyte or its complement by means of the probe, wherein the probe comprises one or more nucleotides and/or nucleotide analogues that have an affinity increasing modification.